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14. ABSTRACT
IGFBP-3 is a potent inducer of apoptosis in both androgen-dependent and androgen-independent prostate cancer lines. When the nuclear receptor RXRalpha was described as an unexpected intracellular binding partner for IGFBP-3 and effects on DNA transcription were demonstrated, rapid effects of IGFBP-3 on programmed cell death (apoptosis) still could not be explained. These rapid effects on apoptosis were clarified when I hypothesized that IGFBP-3 was a biological signal for Nur77 nuclear receptor translocation to the mitochondria where an apoptotic cascade is initiated. We proposed to determine scientifically the protein regions in each of these important cell death molecules that essential for apoptotic action and demonstrate this observation with mouse models. Our data so far reveal a nuclear export sequence in IGFBP-3. Mutation of this sequence impairs its apoptotic activity. Utilizing the IGFBP-3 KO mouse, we show that IGFBP-3's critical role in castration-induced apoptosis. Mating studies are underway to determine the effects of genetically deleting Nur77 and IGFBP-3 in the ontogeny of prostate cancer.

15. SUBJECT TERMS
IGFBP-3, apoptosis, prostate cancer, nuclear receptors

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Introduction

Prostate Cancer (CaP) continues to be the most frequently occurring malignancy (aside from skin cancers), found in American men. IGFBP-3 is a potent inducer of apoptosis in both androgen-dependent and androgen-independent prostate cancer lines. When the nuclear receptor RXRalpha was described as an unexpected intracellular binding partner for IGFBP-3 and effects on DNA transcription were demonstrated, rapid effects of IGFBP-3 on programmed cell death (apoptosis) still could not be explained. These rapid effects on apoptosis were clarified when I hypothesized that IGFBP-3 was a biological signal for Nur77 nuclear receptor translocation to the mitochondria where an apoptotic cascade is initiated. This project will determine scientifically the protein regions in each of these important cell death molecules that essential for apoptotic action and demonstrate this observation with mouse models. The innovative aspects of this grant include: (1) Characterization of a novel interface (i.e. mitochondrial localization) of nuclear receptor / IGFBP superfamilies in the initiation of tumor programmed cell death; (2) Development of pre-clinical mouse models of prostate cancer that can be used to assess therapies that exploit the IGFBP-3:Nur77:RXR cell death pathway; and (3) provide a compelling rationale for Phase I studies of IGFBP-3 (or small molecule mimetics of this pathway) in men with prostate cancer.

Body

Task 1. Characterize IGFBP-3 protein-protein interactions and mitochondrial targeting in vitro and demonstrate that they are essential for IGFBP-3 induced apoptosis.

a. Confirm IGFBP-3/RXR/Nur77 ternary complex formation via protein-protein interaction studies. (Months 1-6)

We have established association as published in our *Carcinogenesis* paper last year and referenced in last year's progress report.

b. Validate a putative nuclear export sequence (NES) in IGFBP-3. (Months 7-9).

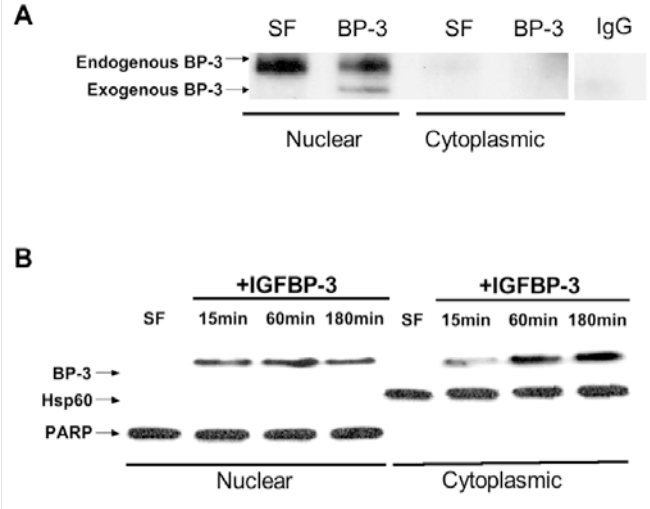

Fig. 1. **IGFBP-3 enters the nucleus rapidly and subsequently is exported to the cytoplasm.** (A) 22RV1 CaP cells treated with IGFBP-3 for 15 minutes, and subcellular fractions were immunoprecipitated and immunoblotted with IGFBP-3. (B) Time Course of IGFBP-3 subcellular localization in R- MEF cells, Hsp60 and PARP were used to assess purity of the subcellular fractions.

Nuclear localization of IGFBP-3 is a well-described phenomenon and has been demonstrated in a variety of cellular models. IGFBP-3 possesses a consensus bipartite nuclear localization sequence, and nuclear transport is facilitated by importin-β factor. We have begun to characterize the intracellular trafficking of IGFBP-3 and its relation to biological function and have recently published mechanisms that are involved in the re-uptake after secretion of IGFBP-3. After internalization by endocytic pathways, IGFBP-3 is first targeted to the nucleus (Figure 1A). 500 ng of rhIGFBP-3 was added to 22RV1 CaP cells for 15 minutes, subcellular fractions were isolated and immunoprecipitated with goat polyclonal anti-IGFBP-3. Immunoprecipitation of treated cell lysate with control IgG is also shown on the right. Proteins were resolved by SDS-PAGE and IGFBP-3 was identified by immunoblotting with mouse monoclonal anti-IGFBP-3. Within 15 minutes of addition of IGFBP-3, we conclude that the initial destination of IGFBP-3 is the nucleus. To expand the time course and explore the IGF-independent intracellular trafficking of IGFBP-3, we utilized IGF receptor-negative (R–) embryonic fibroblast cells (MEFs) derived from an IGF-1R knock-out mouse. These cells have been shown previously to neither bind nor respond to IGFs. R- MEFs were pulsed with 500 ng of IGFBP-3 and subcellular localization was followed over a 180 minute time course (Figure. 1B). To demonstrate the purity of the nuclear fraction, expression of mitochondria-specific protein Hsp60 and nuclear-specific protein poly(ADP-ribosyl) polymerase (PARP) is shown. Again, the primary destination of IGFBP-3 in 15 minutes is the nucleus, after which levels in the cytoplasm begin to increase, consistent with an active export mechanism.

c. Delineate the mitochondrial targeting sequence (MTS) in IGFBP-3. (Months 7-9). **A highly conserved NES and a mitochondrial targeting sequence (MTS) lies within the C-terminal and N-terminal domain of IGFBP-3, respectively.**

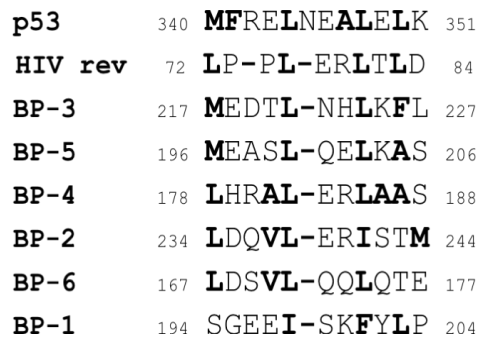

Figure 2. A Nuclear Export Sequence (NES) in IGFBP-3. This region was identified in the C-terminal region of IGFBP-3 between AA 217 and 228 due to similarity to established NES in regions of conserved spacing and hydrophobicity in p53 and HIV rev proteins. Analogous regions of other members of the IGFBP family are shown in descending order of similarity. Hydrophobic AAs are highlighted in **BOLD**.

Because of the preliminary data in Figure 1 showing the shuttling of IGFBP-3 from the nucleus to the cytoplasm, we examined the IGFBP-3 primary amino acid sequence to determine whether it contains a leucine-rich sequence of conserved spacing and hydrophobicity which fits the criteria established for an NES. We observed that the C-terminal residues between amino acids 190 and 201 conform to this motif, as indicated by their similarity to other known NESs such as HIV REV and p53 (Figure 2). In addition, alignment of this sequence across other members of the IGFBP family is shown. This sequence is highly conserved in widely divergent species. In addition to its role as a nuclear transcription factor, p53 has a confirmed pro-apoptogenic role at the mitochondria. This putative sequence suggests extra-nuclear trafficking of IGFBP-3 and the possibility of a mitochondrial function for IGFBP-3.

To further support mitochondrial localization of IGFBP-3, we analyzed IGFBP-3's AA sequence with MitoProt (http://psort.ims.u-tokyo.ac.jp/form.html). This calculates the N-terminal protein region that can support a Mitochondrial Targeting Sequence and the cleavage site. The mitochondrial localization of IGFBP-3 is based on hydrophobicity of its leader sequence. This program predicts import into the mitochondrial matrix and cleavage between AA 13 and 14.

We constructed a mutant NES IGFBP-3:FLAG (C-terminal) fusion consisting of leucine to alanine conversions at residues 197 and 200, since analogous mutations in other NES-containing proteins have been reported to prevent nuclear export. Wild-type or mutant NES:FLAG constructs were cloned in expression vectors; verified by sequencing; and transiently transfected to assess subcellular localization. In addition, the ΔMTS mutant was generated by deletion of the MTS between AA 27 and 40 with preservation of the signal peptide.

Western immunoblotting revealed that anti-IGFBP-3 antibody recognized the mutant NES as well as the ΔMTS protein (Fig 3A). Fractionation of transfected 22RV1 prostate cancer cells into nuclear and cytoplasmic fractions revealed that whereas WT IGFBP-3 had equal distribution between nuclear and cytoplasmic fractions after 48 hours transfection, BP-3 NES mutant shows increased amounts in nuclear versus cytoplasmic fractions compared to WT IGFBP-3 (Fig 3B). Immunoblotting with DNA PKC and Hsp60 were used to assess purity of the nuclear and cytoplasmic fractions respectively. No significant change in subcellular distribution as assessed by these fractionation methods was noted in the ΔMTS IGFBP-3 mutant.

Analysis by indirect immunofluoresencent confocal microscopy correlated with subcellular subfractionation for IGFBP-3 localization with known mitochondria/ER markers (Fig 3C). No known proteolytic sites or N - glycosylation sites in BP-3 are being affected in the Δ MTS mutant.

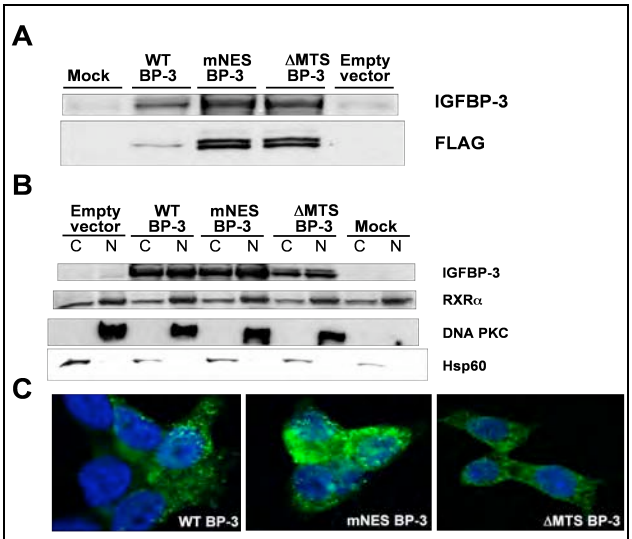

Figure 3. Subcellular localization of IGFBP-3 mutants (A) Recognition of mutant IGFBP-3 by IGFBP-3 antibody. (B) mNES BP-3 accumulates in the nucleus. 22RV1 cells were transfected with WT and mutant IGFBP-3 expression vectors, and fractionated into cytoplasmic and nuclear fractions 48 hours after transfection. BP-3 NES mutant shows increased amounts in nuclear versus cytoplasmic fractions compared to WT IGFBP-3. No known proteolytic sites or N-glycosylation sites in BP-3 are being affected in the Δ MTS mutant. C – cytoplasmic fraction; N – nuclear fraction (C) Immunofluouresence of transfected WT and mutant IGFBP-3 in 22RV1 prostate cancer cells. Green - anti-FLAG (IGFBP-3), blue - DAPI (nuclear staining).

Association of IGFBP-3 with Mitochondria and Endoplasmic Reticulum *in vitro.* In an attempt to obtain more detailed information on IGFBP-3 subcellular protein localization of endogenous IGFBP-3 in 22RV1 prostate cancer cells, we turned to subcellular fractionation via a protocol that combines differential and Percoll gradient centrifugations as this is the preferred method for higher purity fractions [24]. The internal membranes were segregated into mitochondria (Fig 4A, Lane 1), mitochondria-associated membrane (MAM; Lane 2), endoplasmic reticulum (ER; Lane 3), and a pellet from the MAM fraction that was collected at low *g* centrifugation, representing an intermediate zone between mitochondria and MAM fraction (Lane 4). The MAM fraction, a subdomain of the ER, which consists of membrane tubules that provide direct physical contact between the ER and mitochondria, was fractionated to high purity. The presence of IGFBP-3 in the various membrane fractions was assessed by immunoblotting. The relative purity of the fractions was assesed by the presence of specific marker antibodies (Hsp60 – mitochondria; PDI – ER, ACSL4 – MAM). Under baseline conditions *in vitro* IGFBP-3 localizes to the mitochondria (Lane 1), and is even more abundantly represented in the ER and MAM membrane fractions

(Lane 2 and 3 respectively). Bcl-2 expression is detected in all membrane fractions as well as has been previously described. RXRα was detected most prominently in the mitochondria fraction, as well as a faint presence in the MAM. RXRα presence in Lane 4 likely represents mitochondria in this transitional layer. In addition, another binding partner of IGFBP-3, Nur77 was also identified in the MAM and ER, with faint presence in the mitochondrial fraction. Mitochondrial localization of RXRα and Nur77 has been described previously. Immunoflourescence studies revealed co-localization of endogenous IGFBP-3 with Mitotracker and PDI, an ER marker, consistent with the subcellular fractionation studies (Fig 4B).

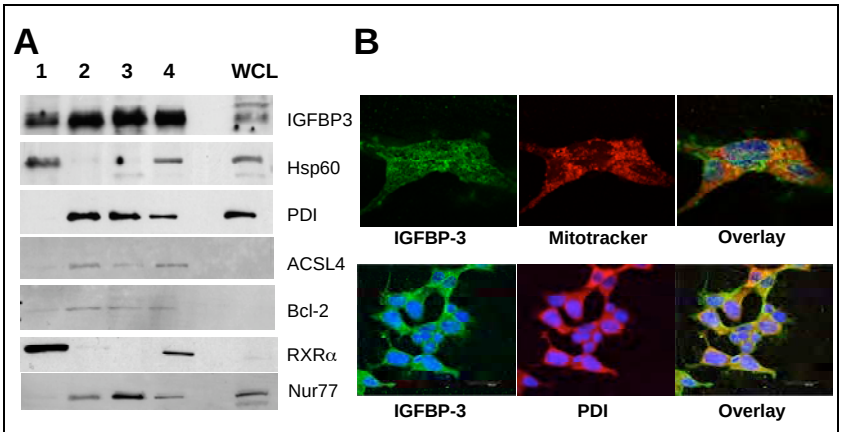

Figure 4. Endogenous Mitochondria and ER Localization of IGFBP-3. (A) 22RV1 prostate cancer cells were fractionated into Lane 1- Mitochondrial Fraction; Lane 2–MAM (Mitochondria Associated Membranes); Lane 3-ER (Endoplasmic Reticulum); and Lane 4–Pellet from MAM. (b) Co-localization (yellow) of IGFBP-3 (green) with Mitochondrial (Mitotracker - red) and ER markers (PDI - red) by immunofluouresence confocal microscopy.

d. *Assess the effects of mutant IGFBP-3 (NES and MTS) on apoptosis. (Months 9-12)*

We have begun to assess the effects of the NES and MTS mutants on apoptosis and show that mutation prevents efficient apoptosis by IGFBP-3. (**Fig. 1**)

Task 2. Define the role of the IGFBP-3/RXR/Nur77 apoptotic pathway in vivo in the TRAMP mouse model.

a. We will age the Nur77 KO and IGFBP-3 KO mice to determine if and when these mice develop prostatic pre-neoplastic lesions. (Months 1-18)
   We have established cohorts and are currently aging them.
b. Examine the role of IGFBP-3 in apoptosis induced by androgen withdrawal by castration of TRAMP and IGFBP-3 KO:TRAMP mice
   i. Develop IGFBP-3 KO:TRAMP cross and assess mouse aging and tumor chronomics. (Months 1-24). Total 100 mice.

   We are currently breeding these mice and genotyping. After some initial problems with mouse mating, we are happy to report that after moving to a new location the mice have resumed breeding.

   ii. Examine subcellular localization of RXR, IGFBP-3, and Nur77 utilizing *in situ* immunohistochemistry and immunoblot post cellular fractionation in

tumors before and after castration (25 mice/group; 13 castration and 12 "sham" castration) at 12 weeks of age (Total 75 mice). Animals to be sacrificed after 6h (2 mice/group) and then every 24h for 4 days. (months 1-6)

iii. Evaluate apoptosis utilizing TUNEL staining and evaluate protein subcellular distribution of IGFBP-3, RXR, and Nur77 by Western blotting. (Months 6-12) (**Fig. 5**).

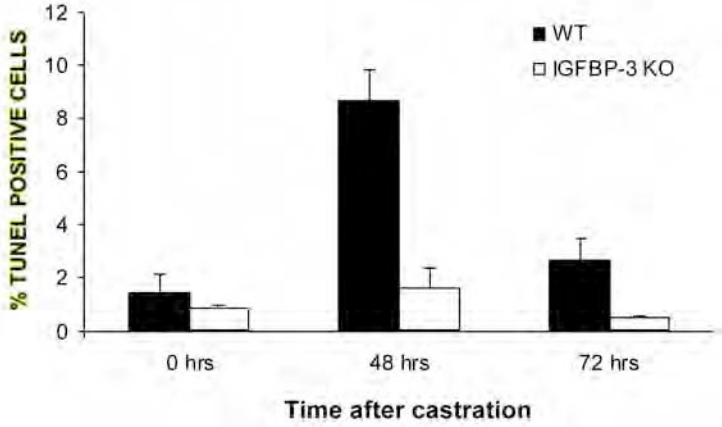

Fig. 5. IGFBP-3 is essential for androgen deprivation induced apoptosis. Twelve-week-old male wild type (WT) mice and IGFBP-3 KO mice were surgically castrated. Prostates were harvested after 48 and 72 hours. Prostates and testes were fixed and stained with H&E for morphologic analysis and quantification of apoptosis using a TUNEL strategy. Apoptosis was quantified by counting the number of TUNEL-positive cells.

WT mice showed a dramatic, 6-fold, increase in the number of TUNEL-positive nuclei at 48 hours post castration. However, IGFBP-3 KO mice prostates failed to show any significant increase in TUNEL staining at 48 hours. By 72 hours TUNEL staining returned to near baseline levels in WT mice and remained near baseline levels in IGFBP-3 KO mice. Serum IGFBP-3 levels were undetectable in the KO mouse and remained unchanged in WT mice post castration. p53 has been shown to be required for prostatic apoptosis, and we have now shown that IGFBP-3, which is activated downstream of p53, is also required for this process. In summary, this is the first description of an *in vivo* role for IGFBP-3 in physiological cell death and indicates that IGFBP-3 is critical for prostatic apoptosis, a fact with potential therapeutic implications in prostate cancer.

c. Study the *in vivo* effects of IGFBP-3 replacement treatment in the IGFBP-3 KO:TRAMP model of prostate cancer. This will commence at a later date after the cross has been established.
   i. Comparison of response to a 4-week course of IGFBP-3 treatment in the TRAMP and TRAMP/IGFBP-3 KO mice on tumor size and histology. (Months 24-30) 7 mice/group total 28 mice (including controls).
   ii. Evaluation of tumor apoptosis by TUNEL staining and proliferation by PCNA staining. (Months 24-30)

iii. Perform immunohistochemistry for subcellular localization of IGFBP-3, Nur77, and RXR as well as subcellular fractionation and immunoblotting for IGFBP-3, RXR, and Nur77. (Months 30-36)

Key Research Accomplishments

- Confirmed IGFBP-3/RXR/Nur77 ternary complex formation via protein-protein interaction studies.
- Defined a Nuclear Export Sequence in IGFBP-3
- Created NES / MTS mutants of IGFBP-3
- Established Mitochondria and Endoplasmic Reticulum Localization of IGFBP-3 in vitro
- Demonstrated Mitochondrial Localization of Recombinant Administered IGFBP-3 to prostate cancer xenografts in vivo.
- Assessed Mutant Effects on IGFBP-3 induced apoptosis
- Castrated IGFBP-3 KO and WT mice
- Demonstrated that IGFBP-3 is essential for androgen deprivation-induced apoptosis
- Continued IGFBP-3 KO:TRAMP mice mating

Reportable Outcomes

Manuscripts Submitted

1. Paharkova-Vatchkova, V, **Lee KW.** 2008 Nuclear Export and Mitochondrial and ER-targeting of IGFBP-3 regulates its apoptotic properties. Submitted. *Neoplasia*.
2. Yamada PM, **Lee KW**. 2008. Perspectives in IGFBP-3 Biology: Local vs. Systemic Action. Submitted *American Journal of Physiology-Cell Physiology.*
3. Hoang PT, Cobb LJ, Park P, Paharkova-Vatchkova V, Cohen P, **Lee KW**. 2008. Delay of diabetes in NOD mice by Humanin. Submitted *Diabetes*
4. Yamada PM, Mehta HH, Hwang D, Powell DR, Hevener A, Cohen P, **Lee KW**. 2008. Genetic Deletion of IGFBP-3 results in peripheral insulin sensitivity and hepatic insulin resistance. Submitted *Cell Metabolism*

Manuscripts in Preparation

1. Paharkova-Vatchkova V, Liu JL, Wang C, **Lee KW.** A BH3-only domain in IGFBP-3 mediates Bax binding and activation.

2. Yamada PM, Mehta HH, Paharkova-Vatchkova V, Lee A, **Lee KW**. Endoplasmic Reticulum Interactions with Grp78 via a BH3-only domain in IGFBP-3 impair insulin-stimulated glucose transport in 3T3L1-adipocytes.

Poster Presentations

Stiehm ER, Lee KW, Hernandez MI, Hoftman AC. Historical Perspective: Newborn illnesses caused by Transplacental Antibodies. Society for Pediatric Research, Honolulu, HI. April 2008.

Hoang PT, Cobb LJ, Cohen P, Lee KW. Humanin Protects beta cells from cytokine-induced apoptosis via ERK and Stat3 activation. Endocrine Society Annual Meeting, San Francisco, CA. June 2008.

Mehta HH, Dwang DL, Sohn JJ, Lue YH, Wang C, Said J, Cohen P, Lee KW. IGFBP-3 is Essential of Prostate Apoptosis Induced by Androgen Deprivation. Endocrine Society Annual Meeting, San Francisco, CA. June 2008.

Yamada PM, Mehta HH, Hwang DL, Cohen P, Lee KW. Genetic Deletion of IGFBP-3 Alters Diet-induced Obesity Associated Inflammatory Markers *in vivo*. Endocrine Society Annual Meeting, San Francisco, CA. June 2008.

Paharkova-Vatchkova V, Lee KW. Identification of a Nuclear Export Sequence (NES) within IGFBP-3 and its Contribution to Apoptosis. Endocrine Society Annual Meeting, San Francisco, CA. June 2008.

Joshi A, Yamada PM, Scherer PE, Lee KW. IGFBP-3 Interacts with Adiponectin and Modulates Reponse to Diet-induced Obesity. Endocrine Society Annual Meeting, San Francisco, CA. June 2008.

Oral Presentations

Yamada P, Lee KW. Novel Insights into Metabolism Utilizing IGFBP-3 KO mice. GRS/IGF 4th International Congress. Genova, Italy. September 2008.

Conclusions

Thus, we conclude that IGFBP-3 is a potent apoptosis inducer with potential implications in prostate cancer. IGFBP-3 induces apoptosis of both androgen-dependent and –independent CaP in vitro, and this has recently been demonstrated in vivo. On a cell biology level, to my knowledge IGFBP-3 is the only molecule known with an endocrine (serum carrier for IGF), as well as an auto-/paracrine function (that can be IGF-independent) with nuclear and extranuclear functions. Therefore, the proposed work shifts the current thinking of IGFBP biology. We have begun to characterize how subcellular localization of IGFBP-3 effects apoptosis induction. In addition, we have for the first time implicated IGFBP-3 in physiologic apoptosis induced by androgen deprivation utilizing the IGFBP-3 KO mouse. Practically speaking, the mechanistic work proposed therein represents the foundation for a new therapeutic intervention in the treatment of men with prostate cancer. These experiments will provide a research-based rationale for clinical trials of IGFBP-3 and establish a role for such therapy in androgen-dependent and –independent prostate cancer. IGFBP-3 has recently undergone successful phase 1 studies in humans and is about to enter phase 2 studies in cancer patients. If successful, these expected findings will improve our understanding of this emerging prostate cancer therapy and facilitate further clinical development in men with prostate cancer.

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- Kim HS, Ali O, Shim M, **Lee KW**, Vuguin P, Muzumdar R, Barzilai N, Cohen P. 2007. Insulin-like Growth Factor Binding Protein-3 induces insulin resistence *in vitro* and *in vivo*. *Pediatric Res*. 61:159-164.

- **Lee KW**, Ma L, Cobb L, Paharkova-Vatchkova V, Liu B, Milbrandt J, Cohen P. 2007. Contribution of the Orphan Nuclear Receptor Nur77 to the Apoptotic Action of IGFBP-3. *Carcinogenesis* 28:1653-1658.

CURRICULUM VITAE

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Fellowship	Pediatric Endocrinology Mattel Children's Hospital at UCLA July 2000-June 2003
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Research Experience

Synthesis and NMR analysis of Lithium organometallic compounds

Karen Hansen, Ph.D.
North Texas State University
June 1985-August 1985

Molecular Genetics of *hsd*R and *hsd*M Restriction-Modification systems in *E.coli K12*

Junichi Ryu, Ph.D.
Loma Linda University
June 1986-August 1986

Sequencing the mHA gene (Bacterial Chemotaxis)

Barry Taylor, Ph.D.
Loma Linda University
June 1987-December 1988

Transcriptional Regulation of the human Insulin-like growth factor binding protein (IGFBP-4) gene

Donna D. Strong
Division of Mineral Metbolism
Jerry Pettis VA MedicalCenter
Loma Linda, CA
June 1989-March 1996

Functional Interactions Between RXR, Nur77, and IGFBP-3 in the Regulation of Cellular Grov and Apoptosis in human carcinoma

Pinchas Cohen, MD
Pediatric Endocrinology
Mattel Children's Hosp at UCLA
Los Angeles, CA
July 2000-June 2003

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	USMLE, Part II	March 1996
	USMLE, Part III	December 1997
	American Board of Pediatrics	October 2000
	American Board of Pediatrics Subboard Endocrinology	August 2005
Medical License	California A065975	
Honors	Magna cum laude	
	Who's Who in American Colleges and Univ.	
	Minority Fellowship in Chemical Res, NTSU	

Summer Fellowship in Biomed Research, LLU
Medical Scientist Training Prog Scholarship
UCLA Department of Pediatrics Res Award

Current Funding

Giannini Foundation Fellow
2001-2003

UCLA CHCRC-K12 Award
2004-2006

UCLA/UCSD DERC P & F
2004-2005

UCLA Stein-Oppenheimer Endowment Award
2004-2005

Lawson Wilkins Pediatric Endocrine Society
Genentech Clinical Scholar
2004-2006

Leadership

American Medical Student Association
President, Loma Linda Chapter (1990-1991)

Memberships

American Academy of Pediatrics
Amer. Assoc. for the Advancement of Science
Endocrine Society
Lawson Wilkins Pediatric Endocrine Society
American Diabetes Association
Society for Pediatric Research

Honors and Awards

1986	Minority Summer Fellowship in Chemical Research, NTSU
1987	Summer Fellowship in Biomedical Research, LLU
1988	Magna cum laude
1989	Who's who in American Colleges and Universities
1990	Medical Scientist Training Program Scholarship
1992	American Medical Student Association, President
2001	Giannini Family (Bank of America) Foundation Fellow
2003	UCLA Department of Pediatrics Fellows Award for Basic Research
2004	Stein-Oppenheimer Award

2004	LWPES/Genentech Clinical Scholar
2005	UCLA / UCSD DERC Developmental Award
2006	UCLA Prostate Cancer SPORE Career Development Award
2007	Phase One Foundation Clinical Scientist Development Award

Publications (peer reviewed)

1. Mohan S, Strong DD, Hilliker S, Malpe R, **Lee K,** Farley J, Baylink DJ. 1993. Dibutyryl cyclic adenosine monophosphate differentially regulates cell proliferation in low and high alkaline phosphatase SaOS-2 human osteosarcoma cells: Evidence for mediation by the insulin-like growth factor-II system. *J Cell Phys.* 156:462-468.

2. Qin X, Morales S, **Lee KW**, Boonyaratanakornkit V, Baylink DJ, Mohan S, Strong DD. Structural and functional analysis of the 5' flanking region of the human insulin-like growth factor binding protein (IGFBP)-4 gene. *Biochimica et Biophysica Acta* . 1350:136-140.

3. Wetterau LA, Moore MG, **Lee KW**, Shim ML, Cohen P. 1999. Novel aspects of the insulin-like growth factor binding proteins. *Mol Genet Metab* 68:161-81.

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10. Ali O, Cohen P, **Lee KW**. 2003. Epidemiology and Biology of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) as an anti-cancer molecule.

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